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13. ABSTRACT ***(Maximum 200 Words)***

Germline mutations of BRCA1 confer an increased risk for breast and ovarian cancer in women and prostate cancer in men. Recent studies suggest that the tumor suppressor activity of BRCA1 is due, in part, to physical/functional interactions with other tumor suppressors, including p53 and the retinoblastoma (RB) protein. Two RB binding sites on BRCA1 were identified, one in the C-terminal BRCT domain and one in the N-terminus, between aa 304 and 394 (Yarden and Brody, *PNAS USA* 96: 4983-4988, 1999; Aprelikova et al. *PNAS USA* 96: 11866-11871, 1999). The N-terminal region of BRCA1 contains a consensus RB binding motif ($^{35-8}$LXCXE), but the role of this site in mediating RB binding and BRCA1/RB functional activity is unknown. Our studies indicate that the BRCA1 interacts with RB, through a binding site between aa 302 and 440, but the binding is not dependent on the LXCXE motif. Nor does the interaction require an intact A/B binding pocket of RB. Transient or stable expression of a wild-type BRCA1 gene (wtBRCA1) caused down-regulation of expression of RB, p107 and p130, associated with a chemosensitivity to DNA-damaging agents. In contrast, expression of an LXCXE-defective BRCA1 mutant (LXCXE → RXRXH) did not cause down-regulation of the RB proteins and the induction of chemoresistance. Our findings suggest that some biologic functions of BRCA1 (eg., chemosensitization) are due, in part, to down-regulation of RB family proteins mediated by an LXCXE site embedded within the N-terminal RB binding site.

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INTRODUCTION

BRCA1. Mutations of BRCA1 (17q21) confer increased risk for breast, ovarian, and prostatic cancers (1-5). Within BRCA1 early-onset breast cancer families, the ratio of ovarian/breast cancers is high for 5' and low for 3' mutations, suggesting possible cell type-specific tumor suppressor activity of the N and C termini of BRCA1 (6). The BRCA1 gene encodes an 1863 amino acid (aa) nuclear phosphoprotein with an N-terminal RING and a C-terminal transcriptional activation domain (TAD) (1,7,8) (Fig. 1). The BRCA1 RING domain mediates interactions with cyclins, cyclin-dependent kinases (CDKs), E2F proteins, a novel C-terminal ubiquitin hydrolase (BAP1), and a novel RING protein (BARD1) (9-12); while the C-terminal minimal TAD interacts with the RNA polymerase II holoenzyme (13), possibly via binding to RNA helicase A (95). BRCA1 also contains both a classic (LXCXE, aa 358-362) (14) and an atypical (LXCXXE, aa 440-445) (15) consensus RB family protein binding motif; but it is not known if and how these putative RB protein binding motifs function in mediating tumor suppression. Unselected invasive breast cancers exhibited decreased BRCA1 mRNA expression (17) and a loss of BRCA1 immunochemical staining (90) compared to non-invasive cancers and benign tissue (17), suggesting a role for BRCA1 in suppressing sporadic breast cancers. While BRCA1 expression suppressed and antisense inhibition of BRCA1 stimulated the growth of adult human breast and ovarian cancers (16,17), BRCA1 may be essential for embryo cell proliferation, since Brca1 (-/-) mice died during early embryogenesis due to a severe defect in cell proliferation (30). Thus, BRCA1 appears to negatively and positively regulate cell proliferation in different contexts.

BRCA1 was implicated in regulation of breast and ovarian cancer cell growth (16,17), cell cycle progression (18-21), apoptosis (22-24), DNA repair (24-28), and maintenance of genomic integrity (29). The mechanisms of these activities are not well understood, but recent studies provide clues. Thus, BRCA1 associates with Rad51, a mammalian DNA recombinase, *in vivo* (25). After DNA damage, both proteins translocate to DNA repair sites (26), implicating BRCA1 in Rad51 pathways of DNA recombination. Brca1 (-/-) mouse fibroblasts are defective in transcription-coupled repair of DNA damage from ionizing radiation (28). We showed that BRCA1 and BRCA2 expression are co-ordinately down-regulated after certain forms of DNA damage (56,57, see APPENDIX). We also showed that unregulated BRCA1 expression confers chemosensitivity, susceptibility to apoptosis, and reduced DNA repair activity in prostate (24) and breast cancer (see preliminary studies). Interestingly, BRCA2 directly interacts with Rad51 (63); and several studies implicate BRCA2 in repair of double-stranded DNA breaks (64,65). This observation may fit with our finding that BRCA2 expression is up-regulated in cells transfected with BRCA1 (24). BRCA1 associates with the C-terminus of p53 via a region mapping to aa 224-500 of BRCA1 and enhances transcription of p53 target genes, including cell cycle inhibitor $p21^{WAF1/CIP1}$ and apoptosis gene Bax (91,92). BRCA1 associates with transcriptional co-activator CBP (93); and we showed that BRCA1 down-regulates expression of Bcl-2 and p300 (a homolog of CBP), two proteins that mediate chemoresistance (24). BRCA1 associates with a protein kinase via a region mapping to aa 329-435; and deletion of this region abolishes growth suppression by BRCA1 (94). The sites of BRCA1 involved in interaction with p53 and BRCA1-associated protein kinase include ^{358}LXCXE. The C-terminal TAD of BRCA1 contains BRCT sequences, a motif found in 40-50 proteins involved in the DNA damage response (97). However, BRCT may not be the only site involved in the DNA damage response, since preliminary studies show that expression of a BRCA1 gene with an LXCXE site mutation confers chemoresistance. The role of LXCXE, LXCXXE, and BRCA1:RB interactions in mediating BRCA1 function is the subject of this proposal.

Rb gene family. The Rb1 gene (13q14) plays major roles in regulation of cell cycle progression, differentiation, and apoptosis. The activated (hypo-phosphorylated) RB1 protein (p105) inhibits cell cycle progression from G1 $\rightarrow$ S, in part, via an interaction between the large A/B pocket of RB1 and the activation domains of E2F family transcription factors, resulting in repression of E2F target genes (reviewed in 31). The cell cycle inhibitory activity of RB1 is regulated via interactions of the standard A/B binding pocket domain of RB1 with the LXCXE motif of target proteins. For example, interactions between RB1 and cell cycle regulatory proteins

(G1/S cyclins and CDKs) and viral oncoproteins (SV40 large T antigen, adenovirus E1A, human papillomavirus E7) inactivate the cell cycle inhibitory activity of RB1 (32-36); while binding of RB1 to an LXCXE-like motif (IXCXE) of histone deacetylase HDAC1 recruits HDAC1 to E2F1 target promoters and mediates transcriptional repression by RB1 (37, 38).

The A/B and C domains are conserved in Rb1 gene family proteins p107 and p130, which also bind to LXCXE and regulate cell cycle-dependent transcription (14, 39-41). Activities of p107 and p130 overlap with but are not identical to RB1; and these proteins may partially substitute for RB1 functions. The standard A/B binding pocket, which regulates the phosphorylation state and cell cycle regulatory activity of RB1, is the site of most tumor-associated Rb1 mutations (31). However, accumulating evidence suggests the existence of distinct classes of Rb1 mutations associated with low versus high penetrance tumor phenotypes (42-44). The existence of mutants that confer high versus low probability of tumor development suggest that some mutant RB1 proteins retain partial wild-type tumor suppressor activity; and several recent studies provide experimental verification of this idea (42, 43). It is tempting to speculate that these mutants differ in the capacity for structural or functional interaction with BRCA1.

Significance. BRCA1 functions in growth control, apoptosis, and DNA damage pathways, but the mechanisms of these functions are unclear. RB1 restricts progression from G1 → S by blocking transcription of genes needed for DNA synthesis, via complex protein interactions. Interaction of the A/B pocket of RB family proteins (RB1, p107, p130) and LXCXE or IXCXE motifs of cell proteins (cyclins, CDKs, HDAC1) modulates transcriptional repression by RB. BRCA1 has typical (LXCXE) and atypical (LXCXXE) RB binding motifs, but the physiologic importance of BRCA1:RB interactions is unknown. Preliminary studies suggest that: 1) expression of mutant BRCA1 defective in the RB binding motif in prostate and breast cancer cells confers an altered phenotype, characterized by increased growth rate, chemoresistance, and resistance to apoptosis; and 2) BRCA1 and RB1 interact *in vivo* and *in vitro*. An LXCXE-mutant BRCA1 differentially suppressed *in vivo* tumor growth in cells with wild-type RB1 (MCF-7) vs mutant RB1 (DU-145), suggesting a role for the LXCXE site and BRCA1:RB1 interaction in tumor suppression. This proposal will test the hypothesis that BRCA1:RB interactions mediate breast cancer suppression. Because RB1 molecular pathways have been dissected in depth, these studies will open a new avenue of research on the role of BRCA1 in molecular carcinogenesis. Thus, it would not be surprising to find that certain BRCA1 mutations are carcinogenic because they disrupt the function of RB1 and circumvent the need for an Rb1 mutation to enable breast cancer growth. We believe that BRCA1 and RB1 collaborate in restricting proliferation and in signalling DNA damage and/or executing an apoptosis program in genetically damaged cells. Knowledge obtained from these studies may lead to novel genetic strategies for breast cancer prevention or treatment.

BODY

Some of the findings obtained in this proposal have summarized in two publications attached in Appendices:

SA1. Effect of disruption of BRCA1:RB interaction on human breast cancer (HBC) cell phenotype.
In SA1, we will: a) confirm and extend preliminary studies by determining how BRCA1-RXRXH alters the phenotype of HBC cells with wild-type versus mutant Rb1; b) determine if these alterations are directly linked to LXCXE by demonstrating similar alterations in cells expressing a BRCA1 gene with a different LXCXE mutation; and c) assess the role of an atypical RB binding motif of BRCA1 (LXCXXE) in modulating HBC phenotype.

SA1-a. Phenotype of HBC cell lines transfected with BRCA1-RXRXH.
1. Isolation of BRCA1-RXRXH, wtBRCA1, and control HBC cell clones.
We have successfully established T47 and MCF-7, two breast cancer cell lines, with stable-transfection of pcBRCA1-385 (=wtBRCA1), pc-mutBRCA1-RXRXH (=BRCA1-RXRXH), and empty pcDNA3 vector (=neo) through selected in G418 as described before (see the PUBLICATION-1). To confirm transgene expression, we used a polyclonal C-20 antibody (Santa Cruz) against the C-terminus of BRCA1, that detects both wtBRCA1 and BRCA1-RXRXH on Western blots (Fig. 7 in the PUBLICATION-1). We also confirmed wtBRCA1 and mutant BRCA1 mRNA expression by semi-quantitative RT-PCR (Fig. 7 in the PUBLICATION-1), as described before by us (24,56,57).

2. Phenotypic characteristics of LXCXE mutant BRCA1 vs wtBRCA1 vs control HBC cell clones.
Rationale. After confirming transgene expression in BRCA1-transfected cell clones, we investigated and compared phenotypic characteristics in BRCA1-RXRXH vs wtBRCA1 vs control (neo) clones, including: *in vitro* growth and cell cycle kinetics, response to cytotoxic DNA-damaging agents, DNA repair capacity, and expression of key cell regulatory proteins that may modulate these processes. For each cell line (MCF-7 cells and Du-145, one prostate cancer cell line that have been established in our preliminary studies), three clones of each clonal were assayed; and each experiment was repeated at least twice. Response parameters (eg., population doubling times, ED_{50}s for drug survival will be compared among the three clonal types. Assays are briefly outlined below.
In vitro proliferation. These studies will tell us if the putative BRCA1:RB interaction affects cell proliferation rates under conditions conducive to rapid growth or under stressful conditions (low serum, clonal density, lack of contact with substrate). We found that BRCA1-RXRXH cells had slightly fast growth rate compared to control-NEO cells under normal growth condition (10% serum DMEM) (SEE Fig. 8a, MS in APPENDICES). Similar results were also obtained under certain stressful conditions, such as low serum.
Cell cycle kinetics. Both BRCA1 and RB1 function in cell cycle check-points, mechanisms that ensure orderly replication of the genome and nuclear/cytoplasmic division (61). Failure of check-points may lead to cytogenetic alterations and/or to altered chemo/radiosensitivity, since different forms of damage are preferentially repaired in different cell cycle compartments. Cell cycle distributions of asynchronously proliferating cells were determined by flow cytometry of propidium iodide-stained nuclei (24). Cell cycle distributions were calculated from the DNA histograms using the MODFIT program. We found that both wtBRCA1 and BRCA1-RXRXH did not significantly affect cell cycle, as shown in Fig. 8b of the PUBLICATION-1.

Response to cytotoxic DNA-damaging agents
BRCA1 may preferentially modulate the response to some agents, but not others, depending upon the particular agent's mechanism of action and type of DNA lesion(s) produced. Thus, we described alterations in BRCA1 and BRCA2 expression only in response to specific DNA-damaging agents (56,57); and Brca1 (-/-)

murine fibroblasts exhibited a defect in transcription coupled repair of DNA damage induced by ionizing radiation but not UV (28). In SA1a, we investigated cellular response to adriamycin (ADR) and camptothecin (CPT), two DNA damaging agents in different transfection cells. Dose-responses for ADR were tested in MTT screening assays, a spectrophotometric assay based on mitochondrial conversion of a tetrazolium salt to formazan (45), over a dose range that yields cell viability values from < 10% to > 90%; and ED_{50}s iso-dose values (ie., dose of agent required to reduce cell viability to 50% of control) was calculated. Differences in viability of BRCA1-RXRXH vs wtBRCA1 vs control (neo) clones in MTT assays were confirmed by colony formation (a measure of reproductive viability) and trypan blue dye exclusion [a measure of cell membrane integrity (56,59)]. As shown Fig. 7b of the PUBLICATION-1, we found that Du-145 cells with wtBRCA1 transfection became more sensitive to cell death and apoptosis caused by ADR and CPT compared to the cells transfected with control pcDNA3 vector. However, Du-145 cells transfected with BRCA1-RXRXH exhibited a significantly resistant to ADR and CPT in Du-145 cells. Similar results were also found in MCF-7 cells (Fig. 7d of the PUBLICATION-1). Furthermore, when wtBRCA1-overexpressed cells were transiently transfected with BRCA1-RXRXH, the chemosensitization caused by wtBRCA1 to ADR was significantly blocked by BRCA1-RXRXH.

Cell death may be due to apoptotic (genetically programmed) and/or non-apoptotic (cell necrosis) pathways. Inhibition of apoptosis-induction pathways may be key events for carcinogenesis, permitting survival of genetically altered cells, and for acquisition of chemo/radioresistance (62). As shown in Fig. 7c and 7d, wtBRCA1 clones of DU-145 and MCF-7 were more susceptible than control (parental/neo) clones to apoptosis induction by ADR and CPT; while BRCA1-RXRXH clones were more resistant to apoptosis induction by the same agents. The striking difference in cell survival and apoptosis in cell lines with unregulated expression of wtBRCA1 compared to the BRCA1-RXRXH mutant suggests a major role for the LXCXE site in activation and/or execution of a survival and apoptosis pathway(s).

Apoptotic DNA was visualized on agarose gels (24,53). ADR and CPT that differentially alter cell viability in BRCA1 vs control transfected cells were tested over a range of agent doses, to allow comparisons at equal doses or equal cell survival. We found that wtBRCA1 clones were more susceptible and BRCA1-RXRXH clones less susceptible than controls to apoptosis induction, these results may reflect two possibilities: 1) the LXCXE site modulates the threshhold DNA damage level required for entry into apoptosis; and/or 2) LXCXE modulates the signaling or repair of DNA damage, resulting in an altered amount of damage signaled to the apoptosis machinery. These possibilities were distinguished by examining the relationship between residual DNA lesions and extent of apoptosis. Using non-proteinizing polycarbonate filters (58), we found that the amount of single-strand (SSBs) and double-strand (DSBs) breaks 24 hr after treatment with ADR (20 μM x 2 hr) and X-rays (12 Gy) were: wtBRCA1 cells >Neo control cells > BRCA1-RXRXH cells.

We also determined tumorigenesis of BRCA1-RXRXH cells in vivo compared with Control-NEO cells and wtBRCA1 cells and found that BRCA1-RXRXH mutation cells grew much fast than Control-NEO cells, wtBRCA1 cells had much slow growth rate in vivo (Fig. 8c of the PUBLICATION-1).

SA1-b. Phenotype of HBC cells transfected with BRCA1 containing another inactivating mutation of LXCXE.

Rationale. The BRCA1-RXRXH mutation presumably inactivates LXCXE-dependent BRCA1:RB interaction(s), but it is possible that this mutation causes other alterations of BRCA1 tertiary structure that cause changes in DNA-damage response unrelated to the LXCXE site. The finding of similar phenotypes in different BRCA1-RXRXH transfected HBC clones does not rule out this possibility, but the finding of a similar phenotype conferred by a different LXCXE mutation would provide more convincing evidence that the observed phenotype is directly related to disruption of the LXCXE site. The goal of SA1-b is to verify the importance of BRCA1:RB interaction by testing the phenotype of cell clones transfected with BRCA1 containing another inactivating mutation of LXCXE.

Site-directed mutagenesis of BRCA1. We used oligonucleotide-directed site-specific mutagenesis of

wtBRCA1 expression plasmid to generate an expression plasmid for BRCA1 with LXCXE deleted (BRCA1 ▲ LXCXE), with the MORPH^{TM} Site-Directed Plasmid DNA Mutagenesis Kit (5 Prime → 3 Prime) and oligoprimer #1. We successfully obtained vectors: pCMV3-BRCA1▲LXCXE, pCMV3-BRCA1▲LXCXXE, and pCMV3-BRCA1-RXRXXH. By transient transfection with these vectors into MCF-7 cells, we found that cells transfected with BRCA1▲LXCXE vector became significantly sensitive to cell death and apoptosis caused ADR compared to cells transfected with control pcDNA3 vector. However, cells transfected with pCMV3-BRCA1▲LXCXXE and pCMV3-BRCA1-RXRXXH did nor show any change in cell survival and apoptosis following treatment with ADR.

SA1-c. Phenotype of HBC cells with BRCA1 containing another of LXCXXE or (LXCXE+LXCXXE). **Rationale**. BRCA1 contains an LXCXE-like site (^{440}LXCXXE) that might participate in a BRCA1:RB interaction (15). However, the role of LXCXXE as a docking site for RB proteins and the significance of an LXCXXE:RB interaction remains to be proven. The goal of SA1-c is to discover if a mutation of LXCXXE confers an altered cell phenotype and this phenotype is similar to that conferred by the LXCXE mutation. These studies will address the specific function of LXCXXE in BRCA1 and the physiologically importance of this site in general. Using site-directed mutagenesis with the MORPH kit, we obtained double mutant vectors: pCMV3-BRCA1▲LXCXE+LXCXXE and pCMV3-BRCA1-RXRXH+RXRXXH. By transient transfection with these vectors into MCF-7 cells, we found that cells transfected with pCMV3-BRCA1 ▲ LXCXE+LXCXXE exhibited similar cell survival to cells transfected with pCMV3-BRCA1▲LXCXE, while cells transfected with pCMV3-BRCA1-RXRXH+RXRXXH also showed no difference to cells transfected with pCMV3-BRCA1-RXRXH. Taken together, the RXRXXH motif in the BRCA1 protein may not play any significant role in modulation of cell survival signaling.

SA2. Protein:protein interactions between BRCA1 and RB family proteins.
Goals. In SA2, we will: a) confirm and extend preliminary studies suggesting *in vivo* association of BRCA1 and RB family proteins (RB1, p107, p130) in HBC cell lines; b) assess the roles of LXCXE, LXCXXE, and other sites in BRCA1:RB interactions; and c) establish the importance of these interactions for transcriptional regulation.

SA2-a. *In vivo* interaction between BRCA1 and RB family pocket proteins (RB, p107, p130) in HBC cell lines. Rationale. In SA2-a, we will extend preliminary studies to investigate the association of BRCA1 with different RB family proteins in HBC cells with wild-type vs mutant Rb1. We will to address two specific issues: 1) does BRCA1 associate with p107 and p130 in HBC cells?; and 2) does *in vivo* association of BRCA1 and RB1 require an intact A/B pocket?. If BRCA1:RB1 association occurs only via LXCXE-like sites, then mutant RB1 proteins with defective A/B pockets should not associate with BRCA1. However, preliminary studies suggest otherwise, since DU-145 mutant RB1 associates with BRCA1 *in vivo* and BRCA1-RXRXH appears to bind RB1 *in vitro*.
BRCA1:RB family protein association by immunoprecipitation (IP) assay. We have assessed BRCA1:RB association by IP of cells with wt vs mutant Rb1 genes. To optimize chances of detecting an interaction, we used low stringency IP conditions and pre-label cell proteins with ^{35}S-methionine to allow sensitive autoradiographic detection (71,72). This procedure requires a second IP (BRCA1 IP → RB1/p107/p130 IP) to verify the identity of proteins of expected M_r, but has the added benefit of allowing detection of other proteins in the BRCA1 immunocomplex. Clues to the identity of these proteins are obtained from the M_r of bands precipitated in stoichiometric quantities along with BRCA1 and RB. The presence of a suspected protein can be confirmed by another IP using an antibody (Ab) specific for that protein. Controls included: 1) pre-incubation of IP Ab ± block [immunizing peptide or *in vitro* translated protein (see below)]; 2) use of another primary Ab for IP; and 3) IP with control Ab (normal mouse IgG or irrelevant Ab). In MCF-7 and Du-145 cells, we have adminstrated physical interaction of BRCA1 and RB1 in vivo. BRCA1-RXRXH did not

affect bindings of BRCA1 to RB1., suggesting that an *in vivo* BRCA1:RB1 interaction does not involve LXCXE or the A/B pocket (Fig. 2 of the PUBLICATION-1)

SA2-b. Role of LXCXE, LXCXXE, and other sites in mediating BRCA1:RB family protein interactions.

The goals of SA2-b are to: 1) identify each of the binding sites for RB1 on the BRCA1 protein; 2) determine if p107 and/or p130 can also bind to these sites; and 3) for each RB1 binding site, determine if the BRCA1:RB1 interaction involves the A/B binding pocket domain as opposed to a different domain of RB1.

Assay of in vitro BRCA1:RB interactions by GST capture

RB1 binding sites on BRCA1. To identify RB1 binding sites, we used GST pull-down assays (34) to examine binding of IVT ^{35}S-methionine labelled BRCA1 proteins to beads coated with GST-RB1 fusion protein. This strategy allowed us to rapidly screen BRCA1 mutants for binding to wt-RB1, since: 1) IVT mutant and wtBRCA1 can be prepared directly from plasmid pcDNA3, using the T7 promoter; and 2) we have GST-Rb1 expression plasmids for wt-Rb1 (pGEX-wtRb1) and two A/B pocket mutants (pGEX-Rb1 ▲ Ex21 and pGEX-Rb1 ▲ Ex22) cloned into pGEX2T1. First, we tested beads coated with GST-RB1 vs GST alone (control) for pull-down of IVT BRCA1 proteins, to establish overall structural requirements (LXCXE plus non-LXCXE dependent) for binding to RB1.

The mutant BRCA1 expression plasmids currently available for testing are illustrated in (Fig. 1 of the PUBLICATION-1). Due to no alteration in phenotype of cells after transfection with mutations in LXCXXE motif, thus, mutant BRCA1 expression plasmids obtained from SA1b and SA1c will also be examined for RB1 binding: BRCA1-RXRXXH, and ▲LXCXXE, RXRXH+RXRXXH, and ▲(LXCXE+LXCXXE).

Binding of p107 and p130 to BRCA1. It is possible that the context (surrounding amino acids) of the LXCXE and LXCXXE sites determine the binding specificity among different RB family members to BRCA1. We utilized GST pull-down assays and found that GST-p107 and GST-p130 could pull down IVT wtBRCA1 (Fig. 2e of the PUBLICATION-1), suggesting p107 or p130 also associate with BRCA1. Further studies regarding BRCA1 binding to p170 and p130 will be proposed in a new proposal.

SA2-C. Role of BRCA1:RB interaction in BRCA1 and RB regulated transcriptional pathways.

Rationale. In preliminary studies of DU-145 cells, we found that the BRCA1-RXRXH mutation abrogated the ability of BRCA1 to: 1) down-regulate mRNA and protein levels of several cell regulatory genes (eg., p300 and Bcl-2); and 2) repress the transcriptional activity of the estrogen receptor (ER-α). These findings suggest that the BRCA1:RB interaction may mediate a transcriptional repression function. This putative repression activity probably does not require the C-terminal TAD of BRCA1, since repression activity of wtBRCA1 vs the TAD-defective mutant insBRCA1 were similar. A recent study indicates that the RB1 protein binds to the androgen receptor (AR) by a ligand-independent interaction; and RB1 functions as a coactivator of the AR in DU-145 cells (73). The studies below are designed to investigate the role of the BRCA1:RB1 interaction in transcriptional repression.

Effect of BRCA1 on RB1 repression capacity and vice versa

Modulation of estrogen receptor (ER-α) activity by BRCA1:RB interaction. Breast cancer suppression by BRCA1 may be due, in part, to inhibition of estrogen (E2) stimulation of mammary epithelia. Preliminary studies suggest wtBRCA1 but not BRCA1-RXRXH represses transcriptional activity of the E2-activated ER-α. These observations were made in cell lines with wt-RB1 (T47D) or mutant RB1 (DU-145) (see PUBLICATION-2), suggesting that p107/p130 can substitute for RB1 or that the contribution of RB1 to BRCA1 repression activity does not require an intact A/B pocket of RB1. These studies will examine the contribution of RB1 to repression of ER-α by BRCA1.

Using the ER-α/ERE-TK-Luc reporter system, we determined that the role of the BRCA1 LXCXE motif

in inhibition of ER-α by determining if BRCA1 transgenes with other LXCXE mutations (δ LXCXE, LXCXE → LXSXE) can mediate repression, and found that BRCA1-RXRXH lost wtBRCA1 function in inhibition of ER-α signaling. To determine if failure of BRCA1-RXRXH to repress ER-α is due to sequestration of RB proteins via a non-LXCXE site, we found that pCMV-Rb1, compared to the control vector, did not affect the ER-α inhibitory activity of BRCA1-RXRXH. We also found the wtBRCA1 repression activity was attenuated by selected adenovirus E1A 12S mutants that bind and inactivate RB family proteins but not p300/CBP (74, 75).

Modulation of RB1 repression of E2F by BRCA1. Inhibition of premature cell cycle progression from G1 → S occurs via binding of hypo-phosphorylated RB1 to the E2F1 TAD and recruitment of RB1 to regulatory regions of E2F target genes (31,33). The RB1:E2F1 interaction does not involve the A/B pocket of RB1, leaving it free to recruit transcriptional repressor HDAC-1 (37,38). Preliminary studies indicate that in DU-145 and T47D, wtBRCA1 fails to inhibit E2F1-mediated activation of an E2F-responsive reporter and sometimes causes increased reporter activity (see manuscript, APPENDIX). This finding was surprising, since wtBRCA1 caused down-regulation of p300 and Mdm-2 (24), which are both E2F1 coactivators (76,77). We speculate that in this context, BRCA1 may sequester RB proteins, so that they cannot repress E2F. To test this hypothesis, we used the assay system of pCMV-E2F1-mediated activation of two E2F-responsive reporters: 1) E2F(CD1)-TK-Luc [which has the E2F site from cyclin D1 (-156 to -133)]; and 2) E2F(AdE2)-TK-Luc (E2F site from adenovirus E2), controlling a minimal thymidine kinase promoter (TK81) and the luciferase gene (78). BRCA1 did not alter pCMV-E2F1-mediated activation of two E2F-responsive reporters. Experiments are still going on to determine if BRCA1 sequesters RB family proteins from E2F with addition of increasing doses of pCMV-Rb1 with a fixed dose of wtBRCA1 vector, and addition of increasing doses of wtBRCA1 with a fixed dose of pCMV-Rb1 should relieve repression by pCMV-Rb1.

Role of HDAC-1 and other repressors in transcriptional repression (TR) by BRCA1. TR is mediated by large multi-protein complexes that often include histone de-acetylases (HDACs), enzymes that convert the chromatin template to a transcriptionally inactive state (72,79-81). RB1 bound to LXCXE of BRCA1 would not be able to recruit HDAC-1, since that interaction involves the A/B pocket domain of RB1 (37,38). However, RB1 bound to a C-terminal site of BRCA1 via an interaction that does not involve the A/B pocket, as suggested by preliminary studies, would presumably remain competent to recruit HDAC-1. HDAC-1 also associates with mSin3A and nuclear receptor co-repressors (72,80); and, interestingly, TR complexes containing HDAC-1 and mSin3A had stoichiometric quantities of an unidentified protein with M_r consistent with BRCA1 (≈ 250 kDa) (72). In addition to our preliminary studies indicating that BRCA1 represses ER-α activity, another study indirectly implicates BRCA1 as a modulator of TR. Thus, BRCA1 interacts, through its C-terminal BRCT domains, with CtIP, a novel protein that interacts with CtBP, a transcriptional co-repressor that is also a cellular target of adenovirus E1A (96). Here, we propose to test the hypothesis that BRCA1 associates with repressor molecules as a potential mechanism of TR.

Recruitment of repressors by BRCA1. We have determined if transcriptional repressor N-CoR associate (co-IP) with BRCA1 in cell lines with wt-RB1 (MCF-7 and T47D) and found that the N-CoR protein could be seen in the BRCA1 IP using IP-WB assay as described in the PUBLICATION-1 (see Appendix), but failed to determined the BRCA1 protein in the N-CoR IP. These findings suggest that BRCA1 may be a important component of a multi-protein TR complex, a potentially important finding in understanding its tumor suppressor mechanism. We also determined whether recruitment of repressor involves an RB1 binding site and requires BRCA1:RB1 interaction.

Role of hBrm/BRG-1 in transcriptional co-regulation by BRCA1 and RB1

Background. BRCA1 and p300/CBP are components of transcriptional complexes containing RNA polymerase II (13,85). These complexes also contain hBrm/BRG-1, homologs of the SWI/SNF yeast coactivators (86,87). The 190 kDa hBrm protein has a helicase-like domain with DNA-dependent ATPase activity, an activation domain (AD) homologous to adenovirus E1A (N-terminus) and E2F1, and a C-terminal

Bromo domain (like p300/CBP). hBrm is of interest because: 1) its AD has an LXCXE site (aa 1290-1294); 2) RB1 interacts with ADs of E2F1 and hBrm; 3) hBrm enhances RB1 repression of E2F1; and 4) hBrm interacts (via a region N-terminal to the ATPase domain) with ER-α (via its C-terminal activation function AF-2) and modulates nuclear receptor activity (88,89). Thus, physical and/or functional interactions with hBrm might modulate transcriptional regulation by BRCA1 and RB1.

We also finished experiments to determine if hBrm can associate with BRCA1 *in vivo* by IP assays (BRCA1 IP → hBrm IB) in HBC cell lines with wt-Rb1 (MCF-7 and T47D). We found there do be a physical interaction between BRCA1 and hBrm. We have just obtained hBrm expression vectors. Experiments are going on to determine if hBrm can either attenuate wtBRCA1 repression of ER activation or rescue the repression activity of BRCA1-RXRXH, using the ER-α/ERE-TK-Luc assay.

Reportable Outcomes

ABSTRACT:

Saijun Fan, Jin Bo Xiong, Yong Xian Ma, Ren-qi Yuan, Qinghui Meng, Itzhak D. Goldberg, Eliot M. Rosen Function Role of the BRCA1 LXCXE Motif in Regulation of RB Family Protein Expression and Cellular Chemosensitivity, but not RB Protein Binding. Presented in *The 92nd Annual Meeting of American Association of Cancer Research*, New Orleans, March, 24-28, 2000.

PUBLICATIONS:

(1) **S Fan**, J Xiong, Y Ma, R Yuan, Q Meng, I D Goldberg and EM Rosen. Disruption of BRCA1 LXCXE motif alters BRCA1 functional activity and regulation of RB family but not RB protein binding. *Oncogene* 20 (35): 4827 - 4841, 2001.

(2) **Fan S,** Yuan R, Ma Y, Meng Q, Wang JA, Goldberg ID and Rosen EM. Mutant BRCA1 genes antagonize phenotype of wild-type BRCA1. *Oncogene*, in press, 2001.

CONCLUSION

The N-terminal site contains a consensus RB binding motif, LXCXE (aa 358-362), but the role of this motif in RB binding and BRCA1 functional activity is unclear. In both in vitro and in vivo assays, we found that the BRCA1:RB interaction does not require the BRCA1 LXCXE motif, nor does it require an intact A/B binding pocket of RB. In addition, nuclear co-localization of the endogenous BRCA1 and RB proteins was observed. Over-expression of wild-type BRCA1 (wtBRCA1) did not cause cell cycle arrest but did cause down-regulation of expression of RB, p107, p130, and other proteins (e.g., p300), associated with increased sensitivity to DNA-damaging agents. In contrast, expression of a full-length BRCA1 with an LXCXE inactivating mutation (LXCXE-->RXRXH) failed to down-regulate RB, blocked the down-regulation of RB by wtBRCA1, induced chemoresistance, and abrogated the ability of BRCA1 to mediate tumor growth suppression of DU-145 prostate cancer cells. wtBRCA1-induced chemosensitivity was partially reversed by expression of either Rb or p300 and fully reversed by co-expression of Rb plus p300. Our findings suggest that: (1) disruption of the LXCXE motif within the N-terminal RB binding region alters the biologic function of BRCA1; and (2) over-expression of BRCA1 inhibits the expression of RB and RB family (p107 and p130) proteins.

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APPENDICES